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Influence of Cooling Rate on Outgrowth of *Clostridium perfringens* Spores in Cooked Ground Beef

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ABSTRACT

The ability of *Clostridium perfringens* spores to germinate and grow was studied to determine a safe cooling rate for cooked beef. Beef samples were inoculated with a cocktail of three strains of heat-shocked *C. perfringens* spores (NCTC 8238, NCTC 8239 and ATCC 10288), vacuum-packaged, and cooked in a stirred water bath to an internal temperature of 60°C in 1 h. Then, samples were cooled through the temperature range of 54.4°C to 7.2°C at rates varying from 6 to 18 h. Samples were removed at various times during cooling to determine if the spores had germinated and multiplied. The samples were plated on tryptose-sulfite-cycloserine agar and incubated anaerobically at 37°C for 48 h. Minimal growth was observed with cooling periods of up to 15 h. However, with the time to achieve 7.2°C extended to 18 h, *C. perfringens* spores germinated and grew from an inoculum of approximately 1.5 $\log_{10}$ to about 6.0 $\log_{10}$ CFU/g. This study indicated that pasteurized cooked beef must be cooled to 7.2°C in 15 h or less to prevent *C. perfringens* foodborne disease outbreaks.

Key Words: *Clostridium perfringens*, spores, beef, cooling rate.

Clostridium perfringens is a continuing concern to the food service industry (*8*). It has been implicated in 11.8% of the total bacterial foodborne disease outbreaks in the United States and 20.6% in Canada (*1,25*). Roast beef, turkey, meat-containing Mexican foods and other meat dishes have been associated with *C. perfringens* food-poisoning outbreaks (4 to 6). Roast beef and other types of cooked beef were implicated as vehicles of transmission for 26.8% of 190 *C. perfringens* enteritis outbreaks in the United States from 1973 to 1987 and 33.9% of 115 outbreaks from 1977 to 1984 (*1,6*). *Clostridium perfringens* spores are a common contaminant of meat, poultry, lentils and many other foods (*15*). Correct cooking reduces pathogenic vegetative cells (*5*); however, heat-resistant spores of *C. perfringens* may survive and receive sufficient heat activation to germinate and multiply in cooked beef and poultry if the rate and extent of cooling is not sufficient. The ability of *C. perfringens* to grow in aerobic environments (*14*) increases chances of foodborne illness in these types of meat products.

In food-service operations, foods are only pasteurized by temperatures and times used to cook or prepare food, and spores of *C. perfringens*, which have a decimal reduction value (D) at 99°C of 26 to 31 min (*3*), can be expected to survive. Food poisoning with *C. perfringens* has been traditionally associated with inadequate cooling practices in retail food operations (*1,6*). While the temperature range for growth of *C. perfringens* is 6°C to 52.3°C (*10,12,22*), rapid growth occurs between 35°C and 48.9°C (*2*). The short generation time of the organism, 7.1 to 20 min in the rapid-growth temperature range, means that after the spore has germinated, fast cooling of foods is critical (*2,14,28*).

The U.S. Department of Agriculture (USDA) safe cooling standard (*27*) for cooked beef, roast beef and cooked corned beef requires that the slowest cooling point be cooled from 48.9°C to 12.8°C in 6 h or less. When the temperature reaches 12.8°C, the cooling will continue to 4.4°C before the meat is boxed. Snyder (*23*) extrapolated this cooling rate to determine the time required to cool beef from 54.5°C to 4.4°C. This results in a cooling time of approximately 11 h. In 1993, the Food and Drug Administration (FDA) recognized that inadequate cooling was a major food safety problem (*9*) and established a recommendation that all food should be cooled from 60°C to 5°C in 6 h or less. However, the FDA did not require a change in the operating performance requirements of refrigerators built to National Science Foundation (NSF) Standard 7 (*16*) and made no allowance for the cooling performance of older commercial refrigerators. Today, there still is no change in the performance requirements of NSF refrigerators. Since there is this performance gap between actual retail food-cooling capability and the 1993 code recommendations, it seemed logical to investigate if the cooling period could be extended beyond 6 h without posing a food safety hazard from outgrowth of *C. perfringens* spores. Our intent was to simulate the cooked roast beef environment in the food industry. Accordingly, the objective of this study was to determine a maximum cooling time for cooked beef containing *C. perfringens* spores that will prevent spore outgrowth and multiplication. In addition, we evaluated the

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usefulness of the model of Ratkowsky et al. (*20*) to represent spore outgrowth data during cooling of meat products.

MATERIAL AND METHODS

Strains and spore suspension

Clostridium perfringens strains NCTC 8238, NCTC 8239 and ATCC 10288 from the culture collection of USDA, Agricultural Research Service (ARS), ERRC maintained as sporulated stock cultures in cooked meat medium (Difco Laboratories, Inc., Detroit, MI) were used. An active culture was prepared in freshly prepared fluid thioglycollate medium, and sporulation was carried out in Duncan and Strong sporulation medium as previously described (*13*). After the spore crop of each strain had been washed twice and resuspended in sterile distilled water, the spore suspensions were stored at 4°C. A spore cocktail containing all three strains of *C. perfringens* was prepared immediately prior to experiments by mixing equivalent numbers of spores from each suspension. This composite of spore strains was heat-shocked for 20 min at 75°C prior to use.

Sample preparation and inoculation

Ground beef, obtained from a local retail market, was placed in a thin layer on plastic trays and sterilized at 121°C for 15 min. The fat was poured off and the meat cooled in a walk-in cooler to an internal temperature of 25°C. The pH of the cooked ground beef was 6.23, as determined using a combination electrode (Sensorex, semi-micro, A. H. Thomas, Philadelphia, PA) attached to an Orion model 601A pH meter. Duplicate 3 g ground beef samples were aseptically weighed into 15 x 22.9 cm sterile Whirl-pak™ sampling bags (Model B736, NASCO Modesto, CA) and inoculated with heat-shocked *C. perfringens* spore cocktail so that the final concentration of spores was approximately 1.5 $\log_{10}$ CFU/g. Thereafter, the bags were manually mixed to ensure even distribution of the organisms in the meat sample. The bags were evacuated to a negative pressure of 1,000 millibars and heat sealed using a Multivac Model A300/16 packaging machine (Germany).

Cooking and cooling procedures

Prior to cooking, two bags were opened, sterile copper-constantan thermocouples were placed at the center of each of the ground beef samples and the bags were resealed. Racks holding the ground-beef samples were fully submerged in 4.4°C water in a water circulating bath (Exacal, Model Ex-251HT, NESLAB Instruments, Inc., Newington, NH). To initiate cooking, the bath temperature was raised in a linear fashion to 60°C within 1 h. This process served to simulate the cooking of rare roast beef. The cooling study was performed through the temperature range of 54.4°C to 7.2°C by adding ice to the stirred water bath at varying rates in order to simulate the desired cooling rate (Table 1). The internal temperature of the samples was constantly monitored by the thermocouples. The readings were measured and recorded by a Keithly-Metrabyte data logger connected to a microcomputer. The thermocouple signal was sampled every second, and the two readings were averaged to determine the sample temperature. The time-temperature schedule for cooling was monitored or controlled by adding ice approximately at each 5 min interval to reduce the temperature according to the desired cooling time. Two replications were performed for the cooking and subsequent time-temperature intervals for cooling.

Enumeration procedure

Samples were removed at the appropriate time intervals based on 6, 9, 12, 15 or 18 h cooling periods to achieve cooling from 54.4°C to 7.2°C. Sterile 0.1% peptone water (3 ml) was added to each bag to give a 1:1 (wt/vol) slurry and macerated for 60 s in a Stomacher Lab-blender™ (Model 400, Spiral Systems, Inc.). An 0.1 ml portion was spread over the surface of tryptose-sulphite-cycloserine (TSC) agar. Also, serial dilutions were made in 0.1% peptone water (wt/vol) followed by spiral plating of each dilution in duplicate on TSC agar without egg yolk enrichment (*11*) and overlaid with TSC agar without egg yolk. The total *C. perfringens* population was determined after 48 h of incubation at 37°C in a Gas Pak™ system (Baltimore Biological Laboratory, Cockeysville, MD).

TABLE 1. *Safe and hazardous cooling times and temperatures for cooked ground beef containing* C. perfringens *spores.*

Elapsed	Cooling-time/temperature	
Time (h)	54.4°C to 7.2°C in 15 h[a]	54.4°C to 7.2°C in 18 h[b]
1	46.4	47.6
2	39.6	41.7
3	33.9	36.6
4	29.1	32.2
5	25.0	28.3
6	21.6	25.0
7	18.7	22.1
8	16.2	19.6
9	14.2	17.4
10	12.5	15.6
11	11.1	13.9
12	9.8	12.5
13	8.8	11.3
14	7.9	10.2
15	7.2	9.3
16	--	8.5
17	--	7.8
18	--	7.2

[a] Safe cooling time.
[b] Hazardous cooling time.

Data processing

Generation time during a changing growth rate was calculated using the method of Willardsen et al. (*28*) between the temperature range from 22.1°C to 15.6°C when the organism grew rapidly. Constants for the growth model were determined by least-squares nonlinear regression using the method of Powell (*19*). Rate equations were integrated numerically using the trapezoidal method.

RESULTS

The fate of the three-strain composite of *C. perfringens* spores in cooked ground beef cooled from 54.4°C to 7.2°C in 12, 15 and 18 h is shown in Fig. 1. No outgrowth of *C. perfringens* spores was observed at 6, 9 (not shown), 12 and 15 h cooling period. *Clostridium perfringens* spores germinated and multiplied when an 18 h cooling time was followed. The total *C. perfringens* population was 3.16 $\log_{10}$ CFU/g after 5 h and the levels reached 6.0 $\log_{10}$ CFU/g by 10 h (15.6°C) with the 18 h cooling procedure. Rapid growth occurred between 22.1°C (7 h) and 15.6°C (10 h); the generation time was 25.5 min.

DISCUSSION

This study investigated the potential for outgrowth of

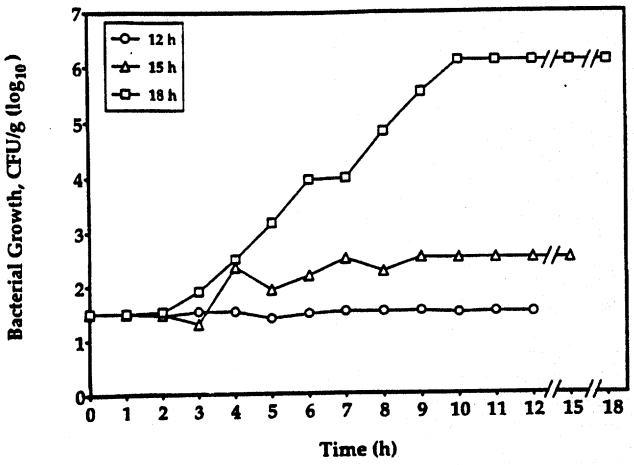

Figure 1. *Fate of 3-strain composite of* C. perfringens *spores in cooked ground beef cooled through the temperature range of 54.4°C to 7.2°C in 12, 15 and 18 h.*

C. perfringens spores in cooked ground beef cooled from 54.4°C to 7.2°C using cooling times varying from 6 to 18 h. When hot cooked food is allowed to cool, the temperature must pass through a range that is favorable for *C. perfringens* spore germination and multiplication of the vegetative cells.

To simulate exponential cooling of cooked foods, the following formulas to calculate the times and temperatures of the hot samples, based on the work of Dickerson and Read (*7*), and Pflug and Blaisdell (*18*), were used. Fourier's law of heat conduction can be expressed as

$$dQ/dt = -kA\,(dt/dx), \qquad (I)$$

where Q is the heat transferred (W), k is the thermal conductivity (W/m-°C) and A is the surface area (m^2). If Newton's law of cooling is applied at the surface, then

$$dQ/dt = Ah_s(T-T_a), \qquad (II)$$

where h_s is a surface heat transfer coefficient (W/m^2-°C) and T_a is the surrounding air or water temperature. By definition,

$$dQ/dt = Vc_p, \qquad (III)$$

where c_p is the specific heat of the substance being heated (J/kg-°C) and V is the volume. Substituting (III) into (II) and integrating gives

$$(T-T_a)/(T_1-T_a) = \exp\,(k_{cool}t) \qquad (IV)$$

$$k_{cool} = Ah_s/Vc_p, \qquad (V)$$

where T_1 is the initial product temperature. The parameter, k_{cool}, can also be determined from the desired experimental cooling rate. If the cooling time (t_c), initial temperature (T_1), end temperature (T_2) and the air or water temperature (T_a) are known, k_{cool} can be computed from:

$$k_{cool} = \frac{\ln\left[\frac{T_2-T_a}{T_1-T_a}\right]}{t_c} \qquad (VI)$$

The temperatures computed for 15 and 18 h cooling periods using the above equations are given in Table 1. It should be noted that the above equations are only approximations; a more detailed analysis of the heat transfer that occurs during roast beef cooling is given by Nolan (*17*).

Hall and Angelotti (*10*) found that *C. perfringens* vegetative cells inoculated into meat began multiplying without any lag phase at 45°C. In a study by Tuomi, Matthews and Marth (*26*), when cooked ground-beef gravy inoculated with a mixture of vegetative cells and spores of *C. perfringens* NCTC 8239 was cooled in a refrigerator, rapid growth of the organism was reported to occur during the first 6 h of cooling when the gravy temperature was in an ideal growth temperature range. While the study by Tuomi, Matthews and Marth (*26*) identified the cooling stage as most critical in assuring the safety of such products, the experimental design included both the vegetative cells and spores of *C. perfringens*; the vegetative cells would not likely be present in a cooked product.

Shigehisa, Nakagami and Taji (*21*) reported on germination and growth characteristics of *C. perfringens* spores inoculated into ground beef at 60°C and cooled to 15°C at a linear cooling rate of 5°C/h to 25°C/h. They observed that the organism did not grow during exposure to falling temperature rates of 25°C/h to 15°C/h. However, multiplication of the organism was observed when the rate was less than 15°C/h. Interestingly, the total population did not change for the first 150 min, regardless of the cooling rate. This study is not totally applicable to typical retail food operations because cooling is not linear; it is exponential.

In the study by Blankenship et al. (*2*), no growth was observed in commercial chili during the first 120 min in isothermal experiments at 37.8, 43.3 and 48.9°C. In our cooling study, we observed similar results with no growth occurring for the first 170 min of the total 18 h exponential cooling time. To simulate commercial chili cooling procedures, Blankenship et al. (2) conducted exponential cooling experiments in which the cooling time was 4 h and 6 h for a temperature decline from 50°C to 25°C. This is approximately equivalent to a cooling rate of 12 h and 18 h for our temperatures of 54.4°C to 7.2°C. They observed a declining growth rate in the case of 4 h and 6 h cooling time. We observed minimal growth during the 15 h cooling period from 54.4°C to 7.2°C. Blankenship et al. (2) heat-shocked their spores, but cooled them to 50°C at room temperature (22°C) before they were exponentially cooled. In our study, the cooling was continuous beginning at 60°C, which is the type of cooling that occurs in most retail food operations. While our study was done with beef, it is likely applicable for other foods contaminated with *C. perfringens* and slow cooling would probably increase the chances of such foods becoming hazardous.

The performance standard for a reach-in refrigerator in the factory, just off the assembly line, tested in a 37.7°C dry-bulb test room, with the refrigerator door never opened for a 4 h period, nothing in the refrigerator, and a 30%

compressor run off-time to allow for defrost, the refrigerator is to hold 3.3°C ± 1.1°C. There is no standard for air velocity. This test standard results in commercial refrigerators that have virtually no cooling capacity. This means that the retail food industry must cool foods in pans < 1 inch deep or put the food in pots in a sink filled with ice, constantly adding ice to the water, and stirring the food every 15 min if it is to cool food to 7.2°C in <4 h (*24*). It is also possible to use a blast-chiller to cool food rapidly. Blast-chill refrigerators can be purchased for $9,000 to $23,000. However, local health departments have not required that operators purchase and use blast-chillers. Many operators have learned to cover and pan food to a thickness of 2 inches, which results in a 12 h to 14 h cooling time to 7.2°C. Based on the results of the present study, we recommend a change in the operating performance requirements of NSF refrigerators.

Using the heat-transfer equations previously discussed and the microbial growth model of Ratkowsky et al. (*20*), a model can be developed to predict the growth of *C. perfringens* during commercial cooling operations. A similar approach was followed by Blankenship et al. (*2*). In the Ratkowsky et al. (*20*) model, the temperature-dependent growth rate ($k_{grow}[T]$) is represented by the following expression:

$$k_{grow}(T) = \{b(T-T_{min})\ \{1 - \exp[c(T-T_{max})]\}\}^2 \qquad \text{(VII)}$$

where T_{min} and T_{max} are experimentally determined minimum and maximum growth temperatures, and *b* and *c* constants fitted by nonlinear regression of experimental growth data. If first-order growth kinetics are assumed, then the bacterial population (N) at any time *t* is given by

$$\ln\left(\frac{N}{N_o}\right) = \int_o^t k_{grow}\,(T[t])dt\ . \qquad \text{(VIII)}$$

where N_o is the initial population of bacteria. We used the data of Willardsen et al. (*28*) for the growth of *C. perfringens* in ground beef to fit the constants *b* and *c*. Optimal values determined by least-squares regression were *b* = 0.00795 and *c* = 0.3951 for T_{min} = 6°C and T_{max} = 52.3°C. The sum of the squares of residuals was 0.00004. A comparison between the predictions of the Ratkowsky model (VII) and the experimentally determined rate constants is given in Fig. 2.

Using the temperatures predicted by equation (IV), equation (III) can be integrated to predict the microbial growth at any time during the cooling period. However, the model does not predict the existence of a lag phase, nor does it accurately predict the bacterial population at temperatures above the maximum or below the minimum growth temperatures. Therefore, we set k = 0 if $T > T_{max}$ or $T < T_{min}$ or $t < t_{lag}$. The lag time (t_{lag}) was determined from the experimental data. The predictions of equation (VIII) are compared to experimentally determined growth curves for 15 h and 18 h cooling times in Fig. 3. In agreement with the findings of Blankenship et al. (*2*) the model was able to accurately represent the growth curves. This analysis demonstrates the validity of the Ratkowsky et al. (*20*) model for representing the growth of bacteria in nonisothermal systems. This model can also be a useful tool for assessing the effect of changing cooling temperatures or cooling times on the outgrowth of *C. perfringens* spores in meat systems.

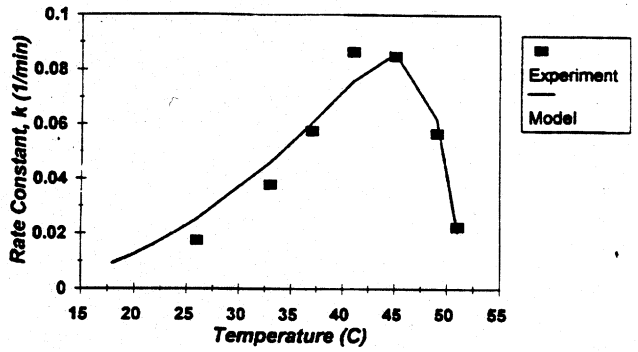

Figure 2. *Experimental* (27) *and predicted rate constants for the growth of* C. perfringens *in cooked ground meat. Model predictions are given by the equation of Ratkowsky et al.* (20) *with b = 0.0079462, c = 0.3951,* T_{min} *= 6 °C and* T_{max} *= 52. 3°C.*

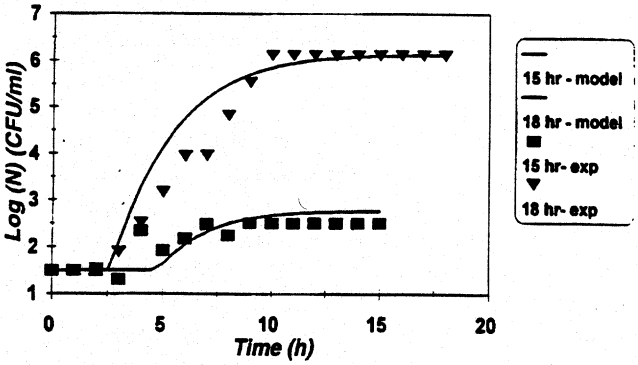

Figure 3. *Experimental and model predictions for the growth of* C. perfringens *in ground meat during cooling. The samples were cooled from 54.4°C to 7.2°C in 15 or 18 h by adding ice to the stirred water bath at varying rates. Coefficients for the model are given in Fig. 2. For the 18 h simulation,* t_{tlog} *= 105 min; for the 15 h simulation,* t_{tlog} *= 285 min.*

CONCLUSIONS

The findings from this study indicate that the spores of *C. perfringens* do not germinate in beef cooled from 54.4°C to 7.2°C in 15 h. However, if the cooling period is extended beyond 15 h, there is a potential for outgrowth to potentially hazardous infective dose levels of >6 $\log_{10}$ CFU/g. In addition, we have shown that the model of Ratkowsky et al. (*20*) is capable of predicting spore outgrowth during cooling.

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